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The lifespan of major histocompatibility complex class I/peptide complexes determines the efficiency of cytotoxic T-lymphocyte responses

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SUMMARY

Major histocompatibility complex (MHC)/peptide association and stability are determined by specific amino acid interactions between peptide antigens and the MHC groove, and are regarded as a critical feature in ensuring efficient monitoring by T cells. In this investigation we examined the relationship between MHC/peptide stability and the immunostimulatory capacity of MHC/peptide complexes. For this purpose we compared synthetic peptide analogues derived from the immunodominant HLA-A11-presented IVTDFSVIK (IVT) epitope, for their capacity to reactivate IVT-specific memory cytotoxic T-lymphocyte (CTL) responses. The analogues differentiated from the wild-type epitope by single amino acid substitution at position 2. All peptides showed similar affinity for HLA-A11 molecules and were recognized by IVT-specific CTL clones, but induced HLA-A11 complexes at the cell surface with different lifespan. This model offered the possibility of comparing the capacity of an immunogenic epitope to stimulate a unique population of T-cell precursors depending on the lifespan of its presentation at the cell surface. We demonstrated that stable HLA-A11/peptide complexes efficiently stimulate IVT-specific CTL responses, while HLA-A11/peptide complexes with short lifespan do not. The precise identification of the role of amino acid residues in the formation of stable MHC/peptide complexes may be relevant for the design of wild-type-derived epitopes with high immunogenicity. These analogues may have important applications in the immunotherapy of infectious diseases and immunogenic tumours.

INTRODUCTION

Major histocompatibility complex (MHC) class I molecules act as receptors for antigenic peptides, 8–10 amino acids long, produced by the intracellular degradation of viral and tumour-derived proteins.[1,2] The formation of MHC/peptide complexes occurs in the endoplasmic reticulum, and the complexes are then transported to the cell surface for surveillance by cytotoxic T lymphocytes (CTL). Peptide association is essential for the formation of stable MHC class I molecules.[3,4] Crystallographic studies revealed that the peptide binding site is localized in a groove formed by the two α-helixes lying across an eight-stranded β-pleated sheet.[1,5] The N and C termini of the peptide form hydrogen bonds with residues lining the highly conserved amino acids at each end of the peptide-binding groove, whereas allele-specific peptide residues, termed anchors, are accommodated in deep polymorphic pockets which exhibit structural and chemical complementarity to the corresponding anchor side chain.[2,6] Anchor residues, usually positions 2 and 9 of the peptide sequence, play a crucial role in high affinity binding, and can determine the stability of MHC/peptide complexe.[7,8] Indeed, we have previously shown that the interactions between anchor positions of peptides and HLA-A11 molecules are highly specific, and determine the efficiency of presentation of immunogenic peptides.[9,10] Stable associations between peptides and HLA-A11 are mediated by amino acids in position 2 carrying methyl or ethyl groups bound to the asymmetric Cβ atom with the correct configuration and by lysine in position 9.[11–13]

The affinity of a peptide for MHC molecules seems to play an important role in determining CTL responsiveness. Indeed, it has been demonstrated that only peptides with a relatively high binding affinity for MHC are immunogenic.[14] Furthermore, it has been shown that the immunogenicity of peptide antigens depends on a low dissociation rate of MHC/peptide complexes,[8,15] and that peptides forming stable complexes represent immunodominant targets of CTL responses.[16]

In this investigation we examine the relationship between human leucocyte antigen (HLA)/peptide stability and the immunostimulatory capacity of HLA/peptide complexes by using synthetic peptide analogues derived from the immunodominant HLA-A11-presented IVTDFSVIK (IVT) CTL epitope. IVT derives from the Epstein–Barr nuclear antigen 4 (EBNA4), amino acids 416–424, and presents high affinity for HLA-A11 molecules, because it sensitizes phytohaemaggluti-

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nin (PHA)-blast to lysis at picomolar concentrations,[17] and induces stable HLA-A11 molecules at the cell surface of the mutant HLA-A11-transfected cell line T2.[12,13] We have recently shown that IVT-peptide analogues carrying the natural and non-natural amino acids Thr, alloThr, Abu, Ile or Leu at anchor position 2 associated with HLA-A11 molecules, but induced HLA-A11 complexes at the cell surface with different stabilities.[13] We have now compared the immunostimulatory capacity of the IVT peptide to that of IVT-analogues in specific peptide-stimulation assays. Our findings indicate that stable HLA-A11/peptide complexes efficiently stimulate IVT-specific CTL responses, while HLA-A11/peptide complexes with short lifespan do not.

MATERIALS AND METHODS

Cell lines

The .174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant .174 LCL with the T-cell line CEM.[18] An HLA-A11 positive subline (T2/A11) was obtained by transfection of a genomic *Hin*dIII fragment containing the HLA-A11 coding sequence.[19] Cell lines were maintained in RPMI-1640 supplemented with 2 mM glutamine, antibiotics, 10% heat inactivated fetal calf serum and 200 μg/ml hygromycin B. PHA-activated blasts were obtained by stimulation of peripheral blood lymphocytes (PBLs) with 1 μg/ml of purified PHA for 3 days and expanded in medium supplemented with interleukin-2 (IL-2), as described.[17]

Peptide synthesis

The IVTDFSVIK (IVT) peptide, corresponding to amino acid 416–424 of the EBV nuclear antigen-4 (EBNA4) and the relative analogues (Table 1), were synthesized by solid phase method using a continuous-flow instrument with on-line UV monitoring. The stepwise syntheses were carried out by Fmoc-chemistry. The fluorenylmethoxycarbonil-4-methylbenzhydrylaminehydrochloride (Fmoc–MBHA) resin was swelled in dimethylformamide (DMF) and packed in the reaction column. Fmoc-amino acids were coupled in a fourfold excess using diisopropylcarbodiimide in the presence of the hydroxybenzotriazole (HOBt). The Fmoc group was cleaved with 20% piperidine-DMF solution. Protected peptides were cleaved from the resin by treatment with modified reagent B (88% trifluoroacetic acid (TFA), 5% H_2O, 7% Et_3SiH) and the resulting products were collected by centrifugation. Crude deprotected peptides were purified by high pressure liquid chromatography (HPLC); purity was $>98\%$. Peptide sequence was confirmed by amino acid analysis and nuclear magnetic resonance (NMR) properties.[12] Peptide stocks were prepared in dimethyl sulphoxide (DMSO) at a concentration of 10^{-2} M and diluted in phosphate-buffered saline (PBS) before use. The alloThr amino acid carries a Cβ with an opposite configuration respect T and consequently the methyl group on Cβ has a different spatial disposition. The Abu amino acid presents a hydrophobic side chain with steric hindrance similar to T and does not present the methyl group on Cβ and C asymmetry in the side chain.

Detection of peptide binding to HLA-A11 molecules by immunofluorescence

Aliquots of 1×10^6 T2/A11 cells were incubated overnight at 37° in 1 ml of serum-free medium (AIM-V) with the indicated concentrations of synthetic peptides. After washing, the cells were stained by indirect immunofluorescence, using the W6.32 monoclonal antibody (mAb) which is specific for HLA-A, -B, -C molecules.[20] Mean fluorescence intensity was determined by fluorescence-activated cell sorting (FACS) analysis.[12]

Detection of HLA-A11/peptide complex stability

Aliquots of 5×10^6 T2/A11 cells were cultured overnight in 5 ml serum-free medium containing 10^{-4} M of the indicated peptides. Then, cells were extensively washed, treated with 50 μg/ml mitomycin C to avoid cell proliferation, divided in aliquots in serum-free medium and maintained at 37° for kinetic experiments. Surface expression of HLA class I molecules was detected at 0, 6, 12, 24, and 36 hr by indirect immunofluorescence using the mouse mAb W6.32, which is specific for HLA-A, -B, and -C molecules. Mean logarithm fluorescence intensity was measured with a FACS analyser. The half-life of HLA/peptide complexes was calculated as the time required for 50% of the molecules to decay.

Preparation of peptide-pulsed cells

Aliquots of 1×10^6 T2/A11 cells were cultured overnight at 26° in 1 ml serum-free medium. Cells were then washed, treated with mitomycin C to avoid cell proliferation, and pulsed with the indicated concentrations of peptides for 3 hr at 37°. After extensive washing the cells were used as stimulators for the reactivation of memory CTL responses.

Generation of CTL cultures

Monocyte-depleted PBLs from the Epstein–Barr virus (EBV)-seropositive donors MF (HLA-A2,11 -B35,51), FB (HLA-A11,24 -B7,44) and PF (HLA-A11,24 -B35,44) were plated in 96-well round-bottom plates at 1×10^5 cells/well, stimulated with T2/A11 cells pretreated overnight at 26°, and pulsed with

Table 1. Peptides

Code	Sequence	Half-life of HLA-A11/peptide complexes (hr)
IVT	H-Ile-Val-Thr-Asp-Phe-Ser-Val-Ile-Lys-OH	30
2T	H-Ile-**Thr**-Thr-Asp-Phe-Ser-Val-Ile-Lys-OH	24
2alloT	H-Ile-**alloThr**-Thr-Asp-Phe-Ser-Val-Ile-Lys-OH	10
2Abu	H-Ile-**Abu**-Thr-Asp-Phe-Ser-Val-Ile-Lys-OH	11
2I	H-Ile-**Ile**-Thr-Asp-Phe-Ser-Val-Ile-Lys-OH	32
2L	H-Ile-**Leu**-Thr-Asp-Phe-Ser-Val-Ile-Lys-OH	8

peptides at different concentrations for 3 hr at 37°, at stimulator : responder ratio 1 : 20. All stimulations were performed in six replicates. The first stimulation was performed in AIM-V medium, and fetal calf serum was added to the cultures after 3 days. A second stimulation was performed in the same conditions on day 7. Starting from day 8 the medium was supplemented with 10 U/ml recombinant IL-2. The specificity of the CTL preparations was investigated at day 14 by testing the cytotoxic activity of each replicate against HLA-A11-positive PHA-blasts treated or not with the IVT peptide. The percentage specific lysis of CTL cultures was determined by the mean of the lysis of each replicate.

Cytotoxicity tests

Cytotoxic activity was assayed in standard 4 hr ^{51}Cr-release assays.[21] PHA-blasts were labelled with 0·1 μCi/10^6 cells of $Na_2{}^{51}CrO_4$ for 90 min at 37°. For the peptide sensitization assays, 4×10^3 PHA-blasts were placed in triplicate wells of 96 V-shaped well plates. Peptides were added to each well, and the plates were incubated for 1 hr at 37° before addition of the effectors.[17] Peptide toxicities were checked in each assay, and were always $\leq 3\%$. Percent specific lysis was calculated as $100 \times$ (c.p.m. sample − c.p.m. medium)/(c.p.m. Triton-X-100 − c.p.m. medium).

RESULTS

Recognition of IVT analogues by IVT-specific CTL clones

In this study we have used the IVT peptide and a group of synthetic analogues (2T, 2alloT, 2Abu, 2I and 2L), differing from the original sequence by single amino acid substitution in position 2 (Table 1). All peptide analogues were previously shown to associate with HLA-A11 molecules. The IVT, 2T and 2I peptides produced stable HLA-A11/peptide complexes for up to 48 hr, with 50% of the complexes still detactable at 30, 24 and 32 hr, respectively (Table 1). In contrast, the 2alloT, 2Abu and 2L peptides dissociate much faster from HLA-A11 molecules. Indeed the relative complexes disappeared after 24 hr and the half-life of the complexes was of 10, 11 and 8 hr, respectively (Table 1).[13]

It is known that slight modifications of MHC-interacting peptide residues can induce conformational changes affecting MHC/peptide recognition by cytotoxic T lymphocytes (CTL) or by specific monoclonal antibodies.[20] To investigate the IVT-peptide analogue recognition by IVT-specific CTL clones, we titrated all peptides on HLA-A11-positive PHA-blasts that were used as targets in cytotoxic assays.

The IVT peptide, corresponding to the immunodominant EBNA4 epitope, induced HLA-A11-restricted target cell lysis at 5×10^{-14} M, while the 2T analogue was active at 10^{-11} M (Fig. 1). The 2Abu and 2alloT peptides induced target cell lysis at levels comparable to the IVT peptide. Similar data were obtained with 2I and 2L peptides (not shown). These results suggest that 2Abu, 2alloT, 2I and 2L peptides are recognized by IVT-specific CTLs as efficiently as the wild-type IVT peptide. The lower recognition of the 2T peptide could be a result of diminished affinity of the peptide for HLA-A11 molecules, or a conformational change of the bound peptide inducing a less favourable orientation of T-cell receptor (TCR)-contacting residues.

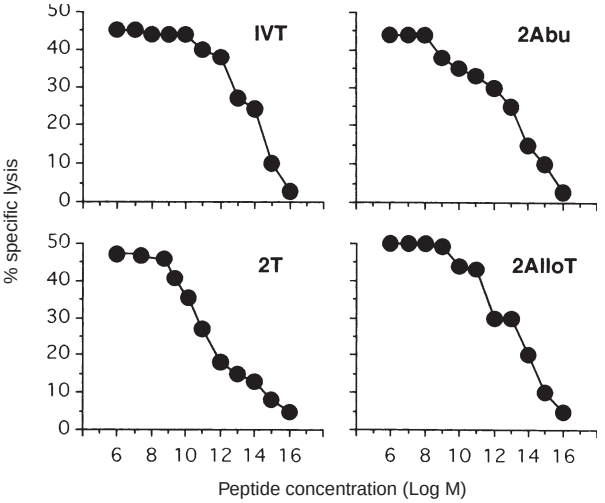

Figure 1. Recognition of IVT analogues by IVT-specific CTL clones. The IVT-specific CTL clone ZAN-43 was tested for its ability to lyse HLA-A11-matched PHA-blasts pulsed with different concentrations of IVT, 2Abu, 2T, and 2alloT peptides before CTL were added to the assay. The percentage specific lysis recorded at 10 : 1 effector/target ratio is shown in the figure.

Affinity of 2T peptide for HLA-A11 molecules

HLA-A11/peptide affinity was assessed by the induction of surface HLA-A11 expression in the T2/A11 mutant cell line.[11] T2/A11 cells were treated overnight at 37° with different concentrations of IVT and 2T peptides in serum-free medium to evaluate the peptide capability of stabilizing empty molecules at the cell surface. After peptide treatment the cells were extensively washed to remove the unbound peptides and the surface expression of HLA class I complexes was evaluated by immunofluorescence using the W6.32 mAb, which recognizes HLA class I molecules independently of associated peptides.

Similar levels of HLA-A11 surface expression were observed on T2/A11 cells pulsed with the different concentrations of peptides, indicating that the 2T peptide associates with HLA-A11 molecules with an efficiency comparable to that of the IVT peptide (Fig. 2). This suggests that the 2T

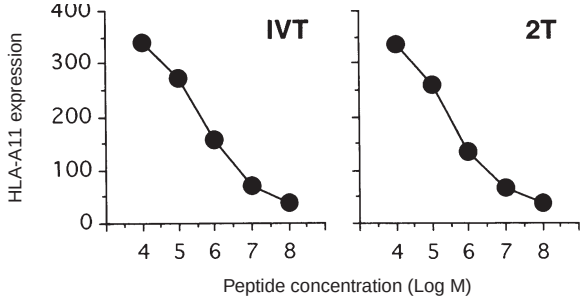

Figure 2. Expression of HLA-A11/peptide complexes at the cell surface. T2/A11 cells were preincubated overnight at 37° with the indicated concentrations of IVT and 2T peptides. Surface expression of HLA class I molecules was detected by indirect immunofluorescence using the W6.32 mAb. Data are expressed as mean fluorescence intensity measured with a FACS analyser. Mean of three different experiments.

peptide has an affinity for HLA-A11 class I molecules comparable to that of IVT, while the lower recognition of the HLA-A11/2T complex by IVT-specific CTLs may be caused by a conformational change of the bound peptide.

Immunostimulatory capacity of IVT-derived peptide analogues

We have previously demonstrated that efficient IVT-specific HLA-A11-restricted CTL responses can be reactivated *in vitro* by stimulation of lymphocytes from HLA-A11-positive EBV-seropositive individuals with T2/A11 cells pulsed with the relevant IVT synthetic epitope.[22] To evaluate the relationship between HLA/peptide stability and the immunostimulatory capacity of antigenic peptides, we performed parallel stimulations of PBLs isolated from the HLA-A11-positive EBV-seropositive donors MF, PF and PA, using T2/A11 cells pulsed with IVT, 2Abu, 2T, 2alloT, 2I or 2L peptides in a range of concentrations from 10^{-6} to 10^{-14} M. After two consecutive stimulations, CTL cultures were tested against HLA-A11-positive PHA-blasts treated or not with the IVT-peptide. Representative results obtained from donor MF are reported in Fig. 3. As previously observed, stimulation with the wild-type IVT peptide efficiently induced IVT-specific CTLs.[22] The IVT-specific lysis of CTL cultures was dependent on the concentration of the stimulating HLA-A11 presented peptide; indeed, high IVT-specific lysis was recorded in CTL cultures stimulated with T2/A11 cells pulsed with 10^{-8} M IVT peptide. The level of specific killing decreased proportionally to the amount of the IVT peptide used in the stimulation, but was still detectable in CTL cultures stimulated with T2/A11 pulsed with 10^{-14} M IVT peptide. CTL cultures obtained by stimulation with T2/A11 pulsed with 2T or 2I peptides showed a similar pattern of activity, even though the IVT-specific cytotoxic activity detected in cultures stimulated with the 2T peptide was lower than that detected in CTL cultures stimulated with the IVT or 2I peptides. It is to be noted that target cells pulsed with the 2T peptide were recognized less efficiently by the TCR of IVT-specific clones than targets pulsed with the wild-type epitope. This may explain the different stimulation efficiency of IVT and 2T peptides.

In contrast, stimulations with T2/A11 pulsed with the 2Abu, 2alloT and 2L peptides did not induce efficient IVT-specific CTL reactivation. Indeed, low IVT-specific killing was detected only in CTL cultures stimulated with the 2Abu and 2L peptides at concentrations 10^{-8}–10^{-10} M. The same levels of killing were obtained with PHA-blasts pulsed with the variant peptides (not shown).

None of the CTL cultures lysed untreated PHA-blasts (Fig. 3).

A similar pattern of responses was observed in peptide stimulations performed with the donors PF and PA (not shown).

These experiments demonstrate that memory T-cell precursors are efficiently reactivated after exposure to MHC/peptide complexes persisting at the cell surface of antigen-presenting cells (APC).

DISCUSSION

Our experimental approach offered the possibility of studying the reactivation of a single CTL population by a single immunogenic epitope, specifically modified in order to obtain different levels of its presentation at the cell surface of APC.

Analysis was performed using the HLA-A11-presented IVT epitope and a group of IVT-analogues carrying a single amino acid substitution at position 2. All peptides showed the same affinity for HLA class I molecules and were recognized by the specific cytotoxic T cells; however, they presented different capacities in producing stable HLA/peptide complexes.

We have demonstrated that the lifespan of MHC/peptide complexes determines the efficiency of presentation and the stimulating capacity of immunogenic peptides. The IVT-

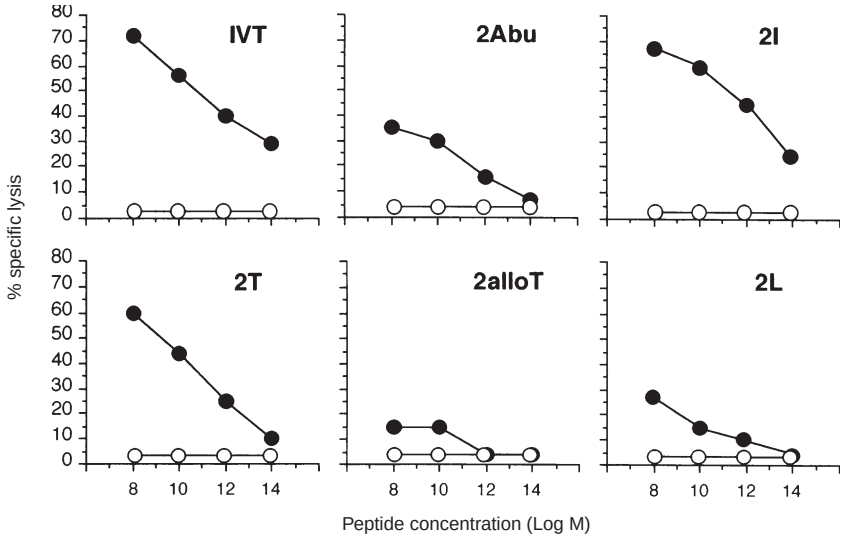

Figure 3. Stimulation of IVT-specific CTL responses by peptides. Freshly isolated lymphocytes derived from the HLA-A11-positive, EBV-seropositive donor MF were stimulated with T2/A11 cells preincubated overnight at 26°, and pulsed with 10^{-8} M, 10^{-10} M, 10^{-12} M, 10^{-14} M of the indicated peptides for 3 hr at 37°. CTL cultures obtained after two consecutive stimulations were tested in cytotoxicity assays against HLA-A11 positive PHA-blasts (open circle) or PHA-blasts pulsed with 10^{-7} M IVT peptide for 1 hr before the assay (closed circle). The percentage specific lysis recorded in one representative experiment is shown in the figure.

analogue that produced stable HLA-A11/peptide complexes induced IVT-specific memory CTL reactivation with an efficiency comparable to stimulations performed with the wild-type epitope. In contrast, the IVT-analogues that produced HLA-A11/peptide complexes with short lifespan demonstrated a poor capacity in IVT-specific CTL reactivation. This suggests that efficient CTL responses to a given epitope require the persistence of MHC/peptide complexes at the cell surface of antigen-presenting cells. Indeed, we have demonstrated that the high immunogenic CTL responses directed to the IVT epitope can be transformed into subdominant responses or non-response by decreasing exclusively the HLA-A11/epitope complex stability.

These data are relevant for the design of wild-type-derived synthetic epitopes suitable for specific immunotherapies. In particular, subdominant epitopes which do not carry proper amino acids necessary for the formation of stable HLA class I complexes, and that induce poor CTL responses, may be transformed into 'improved epitopes' that produce HLA class I complexes stably expressed at the cell surface of APC, able to induce strong CTL responses directed against the subdominant natural epitope.[23,24]

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